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Differential amplification of adenovirus vectors by flanking the packaging signal with *attB/attP*-ΦC31 sequences: Implications for helper-dependent adenovirus production

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Abstract

Current strategies to amplify helper-dependent adenovirus, based on excision of the packaging signal, do not routinely reduce helper adenovirus contamination below 1%. Here, we have tested if reducing the efficiency of the packaging process of the helper adenovirus could impair its packaging without affecting helper-dependent adenovirus production. Interestingly, insertion of *attB/attP*-ΦC31 sequences flanking the packaging signal significantly lengthens adenovirus cycle up to 60 h without reducing virus viability or production yield. This delay occurs in the absence of ΦC31 recombinase indicating that other mechanisms different from excision of packaging signal must be involved. In addition, at 36 h post-coinfection helper-dependent adenovirus are efficiently produced, while production levels of helper *attB/attP*-modified adenovirus are 100–1000 times lower than controls. Therefore, these results suggest that *attB/attP*-mediated packaging impairment of the adenovirus genome is an attractive strategy to significantly reduce helper adenovirus contamination in helper-dependent adenovirus preparations, which in turn would facilitate scaling-up processes for clinical grade preparations.

Keywords: Helper-dependent adenovirus; Recombinase FC31; Adenovirus packaging

Introduction

The introduction of genetic material in target cells requires the use of vectors that package and protect it from the intra- and extracellular degradation, as well as to guide it to the expression site. In this regard, adenovirus (Ad) vectors are widely used in gene therapy clinical trials (http://www.wiley.co.uk/genmed/clinical for more information). To date, most adenoviruses used in preclinical and clinical assays have been deleted in the E1 region or E1/E2 or E1/E4 regions (Amalfitano et al., 1998). However, because adenovirus vectors are highly immunogenic, helper-dependent (HD) adenoviruses, that lack all coding regions from wild-type adenovirus, have been recently developed (for a recent review, see Alba et al., 2005). In spite of their many advantages, HD Ad use in clinical trials is not yet suitable due to low production yields, and the presence of contaminating helper adenovirus generated during their production (Ng and Graham, 2005), especially when high doses must be administered.

The only viral DNA sequences remaining in the HD Ad vector genome (less than 500 base pairs) are those required *in cis* for genome packaging and replication. These sequences are the packaging signal (Ψ) and the inverted terminal repeats (ITRs), the sequences defining the ends of the viral genome (Horwitz, 1990). Thus, generation of HD Ad requires addition of proteins *in trans* (Mitani et al., 1995; Parks et al., 1996). This is achieved through the use of E1-deleted helper vectors. However, viral proteins synthesized in *trans* allow for replication of both helper and HD Ad genomes. Therefore, it is imperative to reduce or

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eliminate the production of helper Ad virions, as well as to develop systems for selective and specific purification of HD Ad. The most common strategy is to flank the Ψ of the helper virus with *lox*P sequences (Chen et al., 1996; Ng et al., 1999). The Ψ is eliminated through a amplification step in a Cre-expressing 293 cell line, thus avoiding packaging of the helper Ad genome without affecting the normal processing of the HD Ad genome (whose Ψ sequence is not flanked by *lox*P sites). However, there are still residual levels of helper virus (0.1% to 1%)(Mitani et al., 1995; Ng et al., 1999). This may be due to escape from Cre-mediated Ψ excision due to limiting Cre-levels (Ng et al., 2002), the acquisition of Cre-resistant mutations and/or to reversibility of Cre reaction allowing reinsertion of the Ψ into the helper vector genome. Efficient regulation of packaging is therefore a key step in the production of high titer pure HD Ad vectors.

To circumvent some of the drawbacks associated with the Cre-mediated approach, we tested another recombinase: ΦC31. In nature, ΦC31 is a unidirectional recombinase that carries out the recombination between the phage (*attP*) site and the host (*attB*) site, which are not identical (Thorpe et al., 2000). This recombination is efficient in *Escherichia coli* and in mammalian cells (Groth et al., 2000), with levels similar or better than Cre and FLP recombinases (Andreas et al., 2002). Interestingly, short fragments (less than 40 pb long) from *attB* and *attP* sequences are still recognized by ΦC31 permitting efficient excision (Groth et al., 2000).

Packaging of the adenoviral genome is a complex process carried out via the interaction of specific viral proteins with the Ψ (Erturk et al., 2003; Ostapchuk et al., 2005). Viral DNA is inserted into the capsid in a sequential process to form a viral particle. The Ψ can be located at either end of adenoviral genome (Hearing et al., 1987), and mutations in Ψ can significantly reduce the efficiency of the packaging process. In addition, the distance between ITR and Ψ is crucial for generating the packaging protein complex: increasing the distance up to 270 bp does not notably affect packaging efficiency, while inserting more than 270 bp, strongly affects the packaging process (Hearing et al., 1987). Moreover, because the sequences flanking Ψ are also involved in recombinase-mediated excision processes during HD Ad amplification, a better knowledge of how this region interacts with viral and cellular proteins is needed.

Thus, taking into account that high levels of Cre expression are toxic in mammalian cells as it induces chromosomal aberrations and increases the number of sister chromatid exchanges (Loonstra et al., 2001; Ng et al., 2002), together with the lack of a direct effect of *lox*P sequences in the packaging process, we analyzed whether the insertion of *attB/attP*-ΦC31 sequences flanking the packaging signal could significantly reduce packaging of helper adenovirus genomes, either by promoting excision of Ψ and/or by disturbing the interaction of Ψ with the packaging complex. Here, we show that flanking Ψ with *attB/attP*-ΦC31 sequences impairs the adenovirus packaging process which, in turn, significantly lengthens the viral cell cycle, but without altering virus viability or production yields.

Results

Generation of attB/attP-modified adenovirus

A family of Ad5 genomes was generated by introducing different combinations of *attB/attP* sequences flanking its packaging signal. All constructs were incorporated into the Ad5 genome through homologous recombination (see Fig. 1). To facilitate tracing of virus amplification kinetics and titering, we inserted a green fluorescent protein cassette (GFP) between the Ψ signal and the *attP* sequence. Thus, we generated the following vectors: Ad5/*attP,* with no *attB* site and just one *attP* site to the right of Ψ (as control for ΦC31); Ad5/FC31.1 that carries *attB/attP* sequences (*attB* sequence to the left of Ψ); Ad5/FC31.2, also containing *attB/attP* sequences, where *attB* is separated from Ψ by a 65-bp spacer sequence (to test the effect of separating *attB* from Ψ), and Ad5/*attB* with no *attP* site and just one *attB* site to the left of Ψ. We used a minimal *attB* region of 53 nucleotides that allows full recombination with the *attP* sequence in the presence of ΦC31 (Groth et al., 2000).

Production of attB/attP-modified adenovirus is affected at 36 h and is not dependent on recombinase ΦC31

For initial production of viral preparations, HEK-293 and HEK-293/FC31 cells (that express ΦC31) were infected with Ad5/Bgal control, Ad5/*attP*, Ad5/FC31.1 or Ad5/FC31.2. Consistently low levels of adenovirus vectors carrying both *attB/attP* sequences were produced during the virus amplification process. Thus, at a standard adenovirus production time (36 h), the amount of infectious Ad5/FC31.1 and Ad5/FC31.2 produced (in IU/cell) in HEK-293/FC31 cells were strongly reduced (>99.9%) compared to control Ad5/Bgal (Fig. 2A). These results indicate abnormalities in the production process that were clearly associated to the presence of both *attB* and *attP* sequences flanking Ψ, regardless if *attB* was close to Ψ or separated by a spacer. However, the presence of *attP* was not sufficient to reduce production levels as Ad5/*attP* behaved as the control vector. Interestingly, in HEK-293 cells the amount of infectious Ad5/FC31.1 and Ad5/FC31.2 produced (in IU/cell)

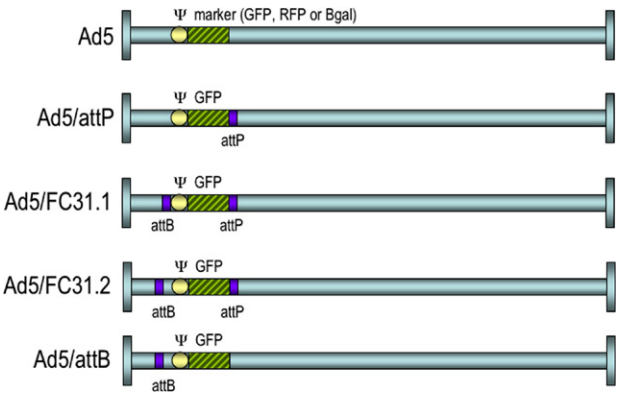

Fig. 1. Adenovirus constructs Ad5/*attP*, Ad5/FC31.1 and Ad5/FC31.2 containing the packaging signal flanked by different combinations of *attB/attP* sequences and a reporter expression cassette.

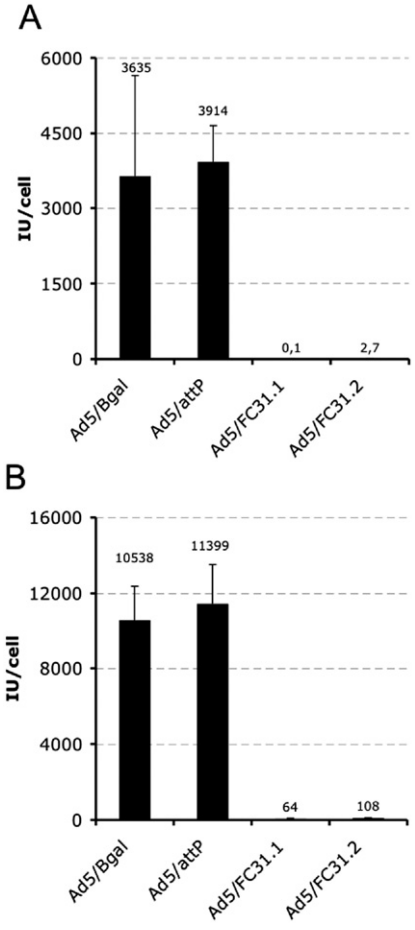

Fig. 2. Viral production levels at 36 h post-infection of Ad5/Bgal, Ad5/*attP*, Ad5/FC31.1 and Ad5/FC31.2 is not dependent on recombinase ΦC31. Newly produced viruses were recovered from crude lysate and titered in HEK-293 cells. IU were determined by end-point dilution assay by quantifying GFP-expressing HEK-293 cells 30 h post-infection. Depending on the vector, data were obtained from 4 to 10 independent experiments. (A) Viral production in HEK-293/FC31 cells. (B) Viral production in HEK-293 cells.

was also strongly reduced (>99%) at 36 h compared to control Ad5/βgal (Fig 2B), indicating that other mechanisms not dependent on the presence of the recombinase must be involved in the low productions levels of *attB*/*attP* vectors. As expected, in HEK-293 cells, Ad5/*attP* also behaved as control Ad5/Bgal. In addition, the same results were observed also in N52.E6 cells indicating that mechanisms are more general and not specific of HEK-293 cells or derivatives (data not shown).

Packaging of attB/attP genomes into viral capside is impaired at 36 h

We then analyzed whether packaging of genomes with Ψ flanked by *attB*/*attP* sequences was affected. HEK-293 cells were infected at an MOI of 5 with a control adenovirus (Ad5/RFP), an *attB*/*attP* containing vector (Ad5/FC31.2) or both together. In this experiment, the Ad5/RFP containing the RFP reporter gene was used to facilitate analysis in coinfection experiments. Moreover, since Ad5/FC31.1 and Ad5/FC31.2 amplify similarly only one (Ad5/FC31.2) was used. At 36 h post-infection, viral genomes were recovered either from infected cells or from virions and quantified by Southern blot using a probe against adenovirus ITRs. As seen in Fig. 3A, viral genomes of Ad5/RFP and Ad5/FC31.2 vectors were produced at the same level when coinfected in the same cells (High Molecular Weight DNA, lane Ad5/RFP+Ad5/FC31.2 and Fig. 3B). In fact, viral DNA production levels were similar either in individual infections (controls) or coinfection. However, analysis of packaged viral genomes in coinfected cells showed that only 1–5% of Ad5/FC31.2 genomes were packaged into virions compared to Ad5/RFP (Fig. 3B), suggesting that low production levels at 36 h were likely due to impaired packaging of genomes with their Ψ flanked by *attB*/*attP* sequences.

Viral cell cycle analysis of attB/attP-modified adenovirus

To test if *attB*/*attP* sequences were impairing the packaging process by affecting packaging kinetics or packaging efficiency, we analyzed production of *attB*-*attP*-modified vectors during the first 72 h (2 standard viral cell cycles). HEK-293 cells were incubated with control and mutant adenovirus at an MOI of 5 and total virus production was determined every 4 h (Fig. 4). Similar to our previous results, production of control Ad5 followed a normal kinetic and reached a plateau after 36 h. Identical results were observed for the Ad5/*attP* vector, which was subsequently utilized as a control vector through the experiments. However, production of Ad5/FC31.1 and Ad5/FC31.2 was slower and reached a plateau about 56–60 h post-infection.

Once we determined that the viral amplification of both *attB*/*attP*-modified adenovirus had a delay of about 20 h with respect to control vectors, we compared whether virus production yield was similar at the end of their viral cell cycle (Table 1). Interestingly, virus yield from Ad5/FC31.2 at the end of its viral cell cycle (60 h) was in the same range of that of the control adenovirus, in contrast with significantly lower levels observed at 36 h (Fig. 2). These data indicated that if we allow completion of viral amplification, virus production efficiency and viability of *attB*/*attP*-adenoviruses are greatly recovered.

Then, to test if the *attB* sequence was responsible for the impairment of the packaging process, an adenovirus containing only the *attB* (Ad5/*attB)* but not the *attP* site was generated. HEK-293 cells were incubated with Ad5/*attB* at an MOI of 5 and total virus production was determined every 4 h (Fig. 4, filled squares). Interestingly, viral cycle of Ad5/*attB* was similar to Ad5/FC31.1 and Ad5/FC31.2 reaching a plateau about 56–60 h post-infection, indicating that, when located at 5′ of Ψ, the *attB* sequence must be responsible for the differential packaging. In addition, as it happens for Ad5/FC31.1 and Ad5/FC31.2, Ad5/*attB* production is very low at 36 h (data not shown) while lengthening of the virus amplification process allowed efficient Ad5/*attB* production (Table 1).

attB-modified vectors allow normal packaging of helper-dependent adenovirus genomes

We finally tested if the delayed virus amplification cycle of *attB*-modified vectors could be utilized for efficient production

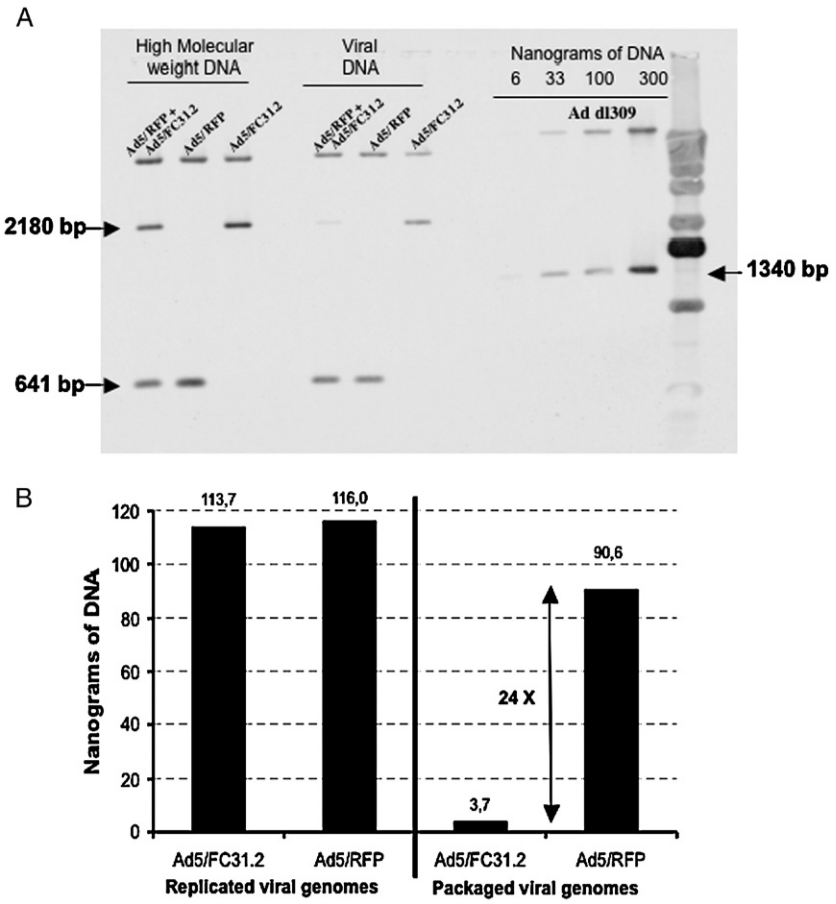

Fig. 3. Analysis of adenovirus packaging. HEK-293 cells were infected with Ad5/RFP or Ad5/FC31.2 at an MOI of 5 IU/cell, or coinfected with both viruses (Ad5/RFP and Ad5/FC31.2) at an MOI of 5 IU/cell per virus. (A) Total nuclear DNA (1 μg), encapsidated viral DNA (100 ng) and different amounts (6, 33, 100 and 300 ng) of control viral DNA (dl309) were separated by gel electrophoresis and analyzed by Southern blot. DNA probe contained the first 194 nt of the adenoviral genome and allows detection of Ad5/RFP (641-bp band) and Ad5/FC31.2 (2180-bp band) genomes. (B) Quantification of intracellular and packaged adenoviral genomes.

of HD Ad vectors. To avoid the potential effects of contaminating helper vectors present in HD Ad preparations, we transfected 293 cells using viral genomes from plasmids instead of transducing from previously amplified viral vector stocks. As HD Ad, Ad5/KCZ, a βgal-expressing vector, was used. Cells HEK-293 were cotransfected with both genomes (pAd5/FC31.2, helper; and pKCZ, HD) at a molar ratio 1:1. Interestingly, after analysis of whole virus sample, virus quantification showed that only infectious HD Ad5/KCZ particles were generated (average of 143.8 IU/10^6 cells), while

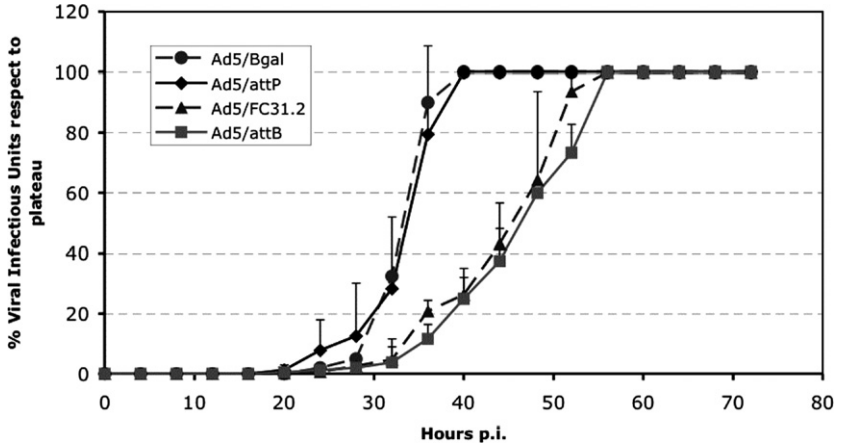

Fig. 4. Viral life cycle of *attB/attP*-modified and control adenovirus. For each adenovirus construct, Infectious Units (IU) were determined every 4 h and compared to the IU value (100%) at the end of each viral life cycle.

Table 1
Viral production levels after viral cycle completion: Ad5/βgal (36 h), Ad5/RFP (36 h), Ad5/*attP* (36 h), Ad5/FC31.1 (56 h) and Ad5/FC31.2 (56 h)

	Adenovirus yield from purified preparations		Ratio IU/PP
	Total IU	Total PP	
Ad5/Bgal	12.9×10^{10}	3.1×10^{12}	1:24
Ad5/RFP	7.1×10^{10}	3.6×10^{11}	1:5
Ad5/*attP*	16.0×10^{10}	5.9×10^{12}	1:37
Ad5/FC31.1	3.6×10^{10}	1.1×10^{12}	1:30
Ad5/FC31.2	2.6×10^{10}	1.6×10^{12}	1:62
Ad5/*attB*	7.1×10^{10}	1.7×10^{12}	1:24

Data obtained from one cesium chloride-purified viral preparation of twenty 15-cm plates. IU were determined by end-point dilution assay by quantifying GFP-expressing HEK-293 cells, 30 h post-infection.

infectious Ad5/FC31.2 particles were undetectable (Fig. 5). Amplified viruses were also recovered 60 h post-transduction (Fig. 5). As expected, at 60 h, infectious helper-dependent Ad5/KCZ virus yield was significantly higher (790 IU / 10^6 cells) than at 36 h. These data demonstrated that *attB*-modified genomes permit packaging and amplification of defective helper-dependent Ad vectors.

Discussion

To date, different strategies have been developed to produce HD Ad vectors, most of them by impairing packaging of the helper adenovirus genome mainly by the use of recombinases (Parks et al., 1996; Sakhuja et al., 2003; Umana et al., 2001). More recently, packaging impairment has been coupled to differential centrifugation by using helper and HD Ad genomes of different size (Sakhuja et al., 2003; Sargent et al., 2004). This system has permitted the reduction of helper contaminating levels down to 0.01–0.1%, which could be the first step to allow their use in human clinical trials. However, the efficiency of Cre and FLPe recombinases is limited due to their toxicity and their reversibility (Loonstra et al., 2001). Similarly, to reduce helper virus contamination in HD Ad preparations, other strategies also target the packaging process by direct mutation of Ψ either by deleting or mutating packaging sequences or a combination of both (Alemany et al., 1997; Kochanek et al., 1996; Soudais et al., 2001). However, none of these strategies is able to routinely reduce helper-contamination below 0.1–1%.

To further impair selective packaging of the helper virus genome, we hypothesized that the recombinase ΦC31 and *attB*/*attP* sequences could be more efficient because it mediates an unidirectional excision, it is active in human cells (Thorpe et al., 2000), and its excision efficiency is similar to Cre and FLPe (Andreas et al., 2002). To study how the packaging process was affected, we flanked the Ψ signal of Ad5 with different combinations of *attB*/*attP* sequences from ΦC31 (Fig. 1). From initial steps of virus amplification (36 h), we observed very low yields in two out of three vector genomes (Ad5/FC31.1 and Ad5/FC31.2) of about 0.5–1% of those observed for controls (Fig. 2). Of note, the Ad5/*attP* vector behaved as the normal control adenovirus suggesting that *attB*, inserted to the left of Ψ (either alone or in combination with *attP*) must be a key factor for low production yields. Surprisingly, these low yields were observed not only in a ΦC31 expressing HEK-293 cell line (Thyagarajan et al., 2000) but also in non-expressing ΦC31 cell lines (HEK-293 and N52.E6), indicating that mechanisms involved were independent on the presence of the recombinase and not restricted to a specific cell line.

Since Ψ was flanked by *attB* and *attP* sequences, we first tested if the packaging process was affected. Comparison between the replicated Ad and packaged Ad genomes revealed that the *attB*/*attP*-modified genomes where equally produced but differentially packaged (Fig. 3), suggesting that an abnormal packaging process could likely be the main cause for low production yields. In agreement with these results, we observed that only *attB*/*attP*-modified vectors clearly lengthened their viral cell cycle (from 36 to 56–60 h) (Fig. 4). However, production yields of *attB*/*attP*-modified vectors were similar to controls when allowed to complete their viral amplification, indicating that only packaging kinetics, but not packaging efficiency, was affected.

Interestingly, only Ad5/*attB* but not Ad5/*attP* has an important delay in the viral cell cycle (Fig 4) indicating that, when located at 5′ of Ψ, the *attB* sequence is able to cause the delay. Different hypotheses could explain differential amplification of *attB*-containing vectors. One possibility is an impairment of the packaging process due to the 53-bp *attB* sequence that we have introduced between left-ITR and Ψ. It is known that the distance between ITR and Ψ can be crucial for an efficient packaging process. However, this seems unlikely since at least 270 or more nucleotides are required to impair packaging (Hearing et al., 1987). In addition, we have not seen any difference between Ad5/FC31.1 and Ad5/FC31.2, which only differ in a 65-bp long spacer between *attB* and Ψ.

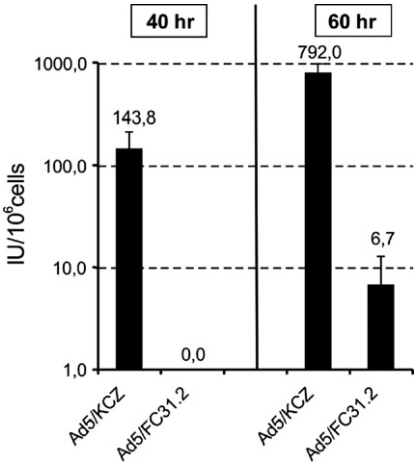

Fig. 5. Differential packaging of *attB*/*attP*-modified genomes versus defective HD genomes. Plasmids pAd5/FC31.2 (*attB*-helper) and pKCZ (HD) were cotransfected into HEK-293 cells at a molar ratio of 1:1. The experiment was performed three times in triplicate and newly produced adenoviruses were harvested 40 h and 60 h post-transduction and quantified by end-point dilution assay.

On the other hand, *attB* may cause conformational changes to DNA and therefore affect binding of the packaging complex to Ψ, thus delaying the packaging process and thereof, lengthening the viral cell cycle. Other possibilities may involve an intracellular factor or a viral protein that may bind the *attB* sequence, hence either impairing direct binding of the packaging complex to Ψ or keeping the adenovirus genome in an abnormal intracellular location away from the packaging complex. Cloning *attB* in other regions of the adenovirus genome may help to determine the mechanisms involved.

Finally, plasmid pAd5/FC31.2 was cotransfected with a plasmid containing a defective HD Ad genome (pKCZ) carrying a βgal cassette (Fig. 5). Of note, at 36–40 h, only infectious particles of HD Ad vector Ad5/KCZ were observed. Not a single infectious particle of *attB/attP*-vector Ad5/FC31.2 could be detected at this time point. Therefore, this experiment clearly shows that an *attB/attP*-containing genome is able to provide viral proteins to allow correct packaging and production of defective HD Ad vector. Surprisingly, the yield of the *attB/attP*-containing genome also was significantly reduced at 60 h after infection in cotransfections with pKCZ. This is in contrast to normal virus yield with the *attB/attP*-containing virus in single infections (Table 1). This result may reflect a competition for packaging between the wild type packaging signal in the pKCZ genome and the *attB*-compromised packaging signal in the *attB/attP*-containing genome. The regulation of viral DNA packaging via competition for limiting trans-acting packaging factors in coinfection experiments was previously suggested based on similar results (Schmid and Hearing, 1997).

In conclusion, we described the generation of adenoviruses containing *attB/attP*-ΦC31 sequences flanking the Ψ signal. These vectors have a delayed viral life cycle, likely caused by the insertion of the *attB*-ΦC31 sequence to the left of Ψ, and show low production yields at 36 h post-infection but normal titers when infections were allowed to proceed for longer periods of times. This is the first time that a protocol is described for the selective regulation of Ad packaging that is not based on recombinase-mediated excision of Ψ but rather on differential packaging kinetics. This system is very efficient in selectively avoiding the packaging of a specific adenovirus genome. For this reason, these results may have profound implications in helper-free HD Ad production, as we can selectively avoid packaging of the helper virus, regardless of the expression of specific recombinases. Large-scale production of HD Ad may be facilitated by controlling the harvesting time of infected cells. Indeed, further experiments like detailed analysis of the different steps of the viral cell cycle, identification of cell proteins interacting with *attB* and/or *attP*, studies of their packaging process in different cell lines (including those expressing recombinase ΦC31) and optimization and full production of HD Ad using *attB/attP*-vectors as helpers may help to characterize and understand the mechanisms involved in the lengthening of the viral cell cycle of *attB/attP*-containing adenovirus, as well as their potential to significantly reduce helper contamination in HD Ad preparations.

Material and methods

Construction of attB/attP containing plasmids

pBCPB+ was kindly provided by Dr. Michele Calos (Stanford University, USA) (Groth et al., 2000), pKP1.4ΔCMV by Dr. Eric Kremer and pTG-6600 by Dr. Olivier Danos. Other plasmids used are pXL-TOPO (Invitrogen, La Jolla, CA, USA), pGEMT-easy (Promega, Madison, WI, USA), peGFP-C1 (BD-Clontech, San Jose CA, USA) and pDSRed2-Mito (BD Biosciences). To delete the multicloning site of pTG-6600, this plasmid was digested with *Eco*RI and *Mfe*I creating 3 fragments of 5310, 616 and 183 base pairs (bp). Fragments 5310 and 616 bp were religated to generate pTG-6600-ΔCMV. pBCPB+ was digested with *Spe*I to obtain the *attP* region (221 bp from ΦC31 bacteriophage), that was cloned into pTG-6600-ΔCMV digested with *Spe*I and *Nhe*I to generate pTG-6600ΔCMVΨ*attP*. pKS-RSV/GFP (donor of GFP cassette) and pTG-6600ΔCMVΨ*attP* were both digested with *Sal*I and *Spe*I to generate pTG-6600ΔCMVΨGFP*attP* (pRAF2.1). Directed mutagenesis was performed in plasmid pTG-6600-ΔCMVΨ*attP* with primers MutDIR*Age*I: 5′-CAC CGG TGT ACA CAG GAA GTG ACA A-3′ and MutREV*Age*I: 5′-CAC CGG TGT ACA CAC CAA AAA CGT C-3′ to introduce an *Age*I site in the nucleotide 189 and generate pTG-6600ΔCMV (*Age*I)Ψ*attP*. *attB*+ *spacer* sequence was cloned into a pGEMT-easy vector to generate pGEMT-*attB.2* by using primers *attB.2*-DIR 5′-TTA TAA AGG TAC CCA CCG GTC CGC GGT GCG GGT GCC AGG GCG TGC CCT TGG GCT CCC CGG GCG CGT ACT CCA CGC GGC CG CAT-3′ and *attB.2*-REV 5′-TTA TAA ACA CCG GTC GCG GCC GCA TAA CTT CGT ATA ATG TAT GCT ATA CGA AGT TAT GCG GCC GCG TGG AGT ACG CGC-3′. pGEMT-*attB.2* and pTG-6600ΔCMV(*Age*I)ΨGFP*attP* were both digested with *Age*I and the 140-bp fragment (*attB.2*) cloned to generate pTG-6600-ΔCMV*attB.2*ΨGFP*attP* (pRAF5.1). Then pRAF5.1 was digested with *Not*I to delete the *spacer* and further religated to generate pTG-6600ΔCMV*attB.1*ΨGFP*attP* (pRAF1.2). To generate pTG-6600ΔCMV*attB.2*ΨGFP (pRAF4.1) *attP* sequence was removed from pRAF5.1. To generate Ad5/RFP, pDSRed2-Mito and pTG-6600 were digested with *Sna*BI and *Eco*RI. A 5685-bp band from pTG-6600 and a 1149-bp band from pDSRed2-Mito were isolated and ligated to generate pTG-6600/RFP (Red Fluorescent Protein). The RFP gene carries a mitochondrial targeting sequence.

Prior to homologous recombination (Chartier et al., 1996), pKP1.4ΔCMV was digested with *Swa*I, while pRAF2.1, pRAF5.1, pRAF1.2, pRAF4.1 and p6600/RFP were all linearized with *Xmn*I and transformed in BJ5183 bacteria to generate the different control and *attB/attP*-modified adenovirus genomes: pAd5/*attP*, pAd5/FC31.1, pAd5/FC31.2, pAd5/*attB* and pKP/RFP. In order to discard any inadvertent secondary mutation, we sequenced every recombinant adenovirus genome from the left ITR to nucleotide 5788 of Ad5, which is the last adenoviral nucleotide of the shuttle vectors involved in the generation of the adenoviral genome by recombination. As expected, we found no mutation/change/variation in any of the

sequences analyzed. Sequences were performed at the Sequencing Service of the Universitat Autònoma de Barcelona, Spain.

Adenovirus generation, production and purification

All adenoviral vectors were produced at the Vector Production Unit in the Center of Animal Biotechnology and Gene Therapy at the Universitat Autònoma of Barcelona (Barcelona, Spain). HEK-293 cells (Q-BIOgene, Montreal, Canada) were grown in DMEM medium (#E15-810, PAA laboratories, Linz, Austria) and 10% of fetal bovine serum (FBS) (PAA laboratories, Linz, Austria) and transfected with Polyethylenimine (PEI, 25 kDa, Aldrich, St. Louis, MO, USA) (2.25 μl PEI 10 mM per 1 μg of DNA), with different linear *Pac*I-digested plasmids containing adenoviral genomes. The initial transfection step was allowed to proceed until viral foci were observed: 3 days for pAd5/*attP* or 8–10 days for pAd5/FC31.1 and pAd5/FC31.2. Virus stocks were sequentially amplified until twenty 15-cm plates were reached, allowing 36 h per step for Ad5/Bgal and Ad5/*attP* and 60 h per step for Ad5/FC31.1 and Ad5/FC31.2. In all cases, the cell pellet was concentrated to a final volume of 40–45 ml, and three freeze/thaw rounds were performed to liberate virus particles. Supernatant was centrifuged in two consecutive $CsCl_2$ gradients [(a) step gradient of 1.40 g/cm^3–1.25 g/cm^3; and (b) an isopycnic gradient of 1.35 g/cm^3] to purify viral particles and desalted using a Sephadex PD-10 desalting column (Amersham Biosciences, Uppsala, Sweden) to remove $CsCl_2$. Final purified viral stocks were titered by determining their concentration (particles/ml) by optical density at 260 nm (1 OD_{260} unit = 1×10^{12} particles/ml), and their infectivity (infectious units/ml) was measured by end-point dilution assay. Briefly, end-point dilution assays were performed in triplicate by infecting 293 cells with serially diluted vectors, and then counting the number of transgene (GFP or βgal) expressing cells after 24 h (Zabner et al., 1999). The particle to infectious unit ratio of adenovirus used in the experiments had an average of 40:1.

Viral production assay

HEK-293 cells were infected with Ad5/Bgal, Ad5/*attP*, Ad5/FC31.1 or Ad5/FC31.2 at 5 IU/cell in a 6-well plate. Samples were washed 6 h later with fresh DMEM medium and pellet and supernatant harvested at 36 and 56 h p.i. Virus particles were liberated and titer calculated at each time point by end-point dilution assay.

Analysis of adenovirus packaging

HEK-293 cells were infected with Ad5/RFP or Ad5/FC31.2 in 5 × 15 cm plates at an MOI of 5 IU/cell. For coinfection experiments, HEK-293 cells were coinfected with both viruses (Ad5/RFP and Ad5/FC31.2) at an MOI of 5 IU/cell per virus. Infected cells were harvested at 36 h post-infection. Most of the cell suspension sample (94%) was used to prepare encapsidated viral DNA following the protocol described by Gräble and collaborators (Grable and Hearing, 1990). The rest of the sample (6%) was used to isolate total nuclear DNA by lysing the cells with Nonidet P-40 (0.6%) and precipitating the nuclei. Total nuclear DNA (1 μg) and encapsidated viral DNA (100 ng) were digested with *Spe*I, separated by gel electrophoresis and analyzed by Southern blot. In addition, we used different amounts (6, 33, 100 and 300 ng) of control viral DNA (dl309) (Jones and Shenk, 1979) digested with *Xba*I to quantify sample DNA. As a probe for Southern blot analysis, a sequence containing the first 194 nt of the adenoviral genome was labeled with ^{32}P by random primer method (Feinberg and Vogelstein, 1983). Viral genomes were analyzed using a PhosphorImager (ABI Storm 680), ImageQuant software and quantified by comparing with control viral DNA from dl309. Adenovirus packaging efficiency was calculated as the number of copies of *attB*-modified adenovirus (Ad5/FC31.2) divided by the number of copies of control Ad (Ad/RFP) in coinfection experiments.

Determination of viral life cycle

HEK-293 cells were infected with control Ad5/Bgal, Ad5/*attP* and Ad5/FC31.2 at 5 infectious units per cell (also at 1 and 0.2 infectious units per cell, data not shown), at 70 to 80% confluency in a 24-well plate. Samples were recovered (pellet and supernatant) every 4 h, up to 72 h. Three freeze/thaw rounds were performed to liberate virus particles and number of IU/cell at each time point calculated by end-point dilution assay. Viral life cycle was defined as the process from virus entry into the cell to formation of infective virus particles. End of viral life cycle was defined as the time when the IU/cell number reached a plateau (values do not increase or even decrease compared to values of previous time points), after a period of maintained exponential amplification of at least 12 h. To avoid the effects of a second amplification cycle, once the end of a viral life cycle was defined for a given vector, further time points were considered as plateau.

Cotransfection assay

HEK-293 cells in a 6-well plate were transfected with 3 μg of defective HD adenoviral plasmid pKCZ and 3 μg of helper plasmid pKP/FC31.2 in triplicate with PEI in serum-free media. Four hours later, the media were replaced by media with 10% FBS. Cells and media were harvested at 40 and 60 h post-transfection and total IU of helper Ad5/FC31.2 and helper-dependent Ad5/KCZ were determined (in the whole viral sample) by end-point dilution assay using fluorescence microscope and X-gal staining, respectively.

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